Candida albicans Pmr1p, a secretory pathway Ca$^{2+}$/Mn$^{2+}$ P-type ATPase, is required for glycosylation and virulence

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SUMMARY

The cell surface of *Candida albicans* is the immediate point of contact with the host. The outer layer of the cell wall is enriched in highly glycosylated mannoproteins that are implicated in many aspects of the fungus-host interaction. Glycosylation of cell wall proteins is initiated in the endoplasmic reticulum and then elaborated in the Golgi as the protein passes through the secretory pathway. Golgi-bound mannosyltransferases require Mn$^{2+}$ as an essential co-factor. In *Saccharomyces cerevisiae* the P-type ATPase Pmr1p transports Ca$^{2+}$ and Mn$^{2+}$ ions into the Golgi. To determine the effect of a gross defect in glycosylation on host-fungus interactions of *C. albicans* we disrupted the *PMR1* homolog, *CaPMR*. This mutation would simultaneously inhibit many Golgi-located, Mn$^{2+}$-dependent, mannosyltransferases. The *Capmr1Δ* null mutant was viable *in vitro* and had no growth defect even on media containing low Ca$^{2+}$/Mn$^{2+}$ ion concentrations. However, cells grown in these media progressively lost viability upon entering stationary phase. Phosphomannan was almost completely absent and O-mannan was severely truncated in the null mutant. A defect in N-linked outer chain glycosylation was also apparent, demonstrated by the under-glycosylation of surface acid phosphatase. Consistent with the glycosylation defect, the null mutant had a weakened cell wall, exemplified by hypersensitivity to Calcofluor White, Congo Red and hygromycin B, and constitutive activation of the cell integrity pathway. In a murine model of systemic infection the null mutant was severely attenuated in virulence. These results demonstrate the importance of glycosylation for cell wall structure and virulence of *C. albicans*.  

Words (246)
INTRODUCTION

*Candida albicans* is the most common fungal agent of invasive disease in humans (1, 2). It is responsible for superficial epithelial infections and, in the immunocompromised host, life-threatening systemic infections (3, 4). The cell wall of *C. albicans* is the immediate point of contact between the fungus and host, and hence is important in host-fungal interactions. The cell wall is comprised of an inner layer of structural polysaccharides, β1,3- and β1,6-glucan and chitin, and an outer layer that is enriched for mannoproteins (5, 6). The highly glycosylated mannoproteins play important roles in adhesion, antigenicity, and in the modulation of the host immune responses (7-11). Both the carbohydrate epitopes and the protein components have been implicated in these roles (7, 8, 12), although the exact epitopes involved are still unclear. The study of glycosylation in *C. albicans* will therefore inform our understanding of the host-fungus interaction. To determine the role of glycosylation in virulence of this fungus we deleted the Golgi P-type ATPase, which transports divalent cations into the Golgi, where they act as essential cofactors for mannosyl transferases.

In *Saccharomyces cerevisiae* glycosylation is initiated in the endoplasmic reticulum by the transfer of the first mannose residue to serine or threonine in *O*-linked glycosylation (13), and transfer of the *N*-linked core structure to asparagine residues (14). The construction and transfer of the *N*-linked core (14, 15) and the initiation of *O*-linked glycosylation are essential processes (16). Glycosylation is continued in the Golgi with the extension of the linear *O*-linked glycans (17) and the extensive elaboration of the branched outer chains of the *N*-linked glycans (18).

The process of glycosylation has been extensively studied in *S. cerevisiae* (13, 14, 15, 17), and this has provided a resource for understanding glycosylation in *C. albicans*. However, key differences exist between the *O*- and *N*-glycan structures present in *S. cerevisiae* and *C. albicans*. For example, in *C. albicans* the terminal *O*-linked glycans that are attached by α1,2-linkages as opposed to α1,3-linkages in *S. cerevisiae* (19), and β1,2-linked mannose residues are present in both the acid-labile and acid-stable *N*-linked glycans of *C. albicans* (20). Following the completion of the *C. albicans* genome project it has also become clear that there has been expansion and contraction of the number of
genes in different mannosyltransferase gene families of *C. albicans* (Bates, Munro, Gow unpublished). Hence, the process of glycosylation has adapted in *C. albicans*, perhaps as a result of selective pressure from its interaction with a mammalian host.

To date, few genes involved in glycosylation in *C. albicans* have been analyzed in detail. Those studied include members of the *PMT* and *MNT* gene families, all of which act in O-glycosylation, and the studies have demonstrated the importance of O-glycans in virulence (19, 21-24). Also, Mnn9p is involved in extension of the N-linked glycan outer chain and hence required for normal cell wall composition (25). Phosphomannan has been implicated in the interaction of *C. albicans* with macrophages. However, deletion of the *C. albicans* *MNN4* gene, which is required for mannosyl phosphate transfer, demonstrated that phosphomannan is not required for macrophage interactions or virulence of *C. albicans* (26). Genes such as *CaVRG4* and *CaSRB1*, which encode proteins required for supplying the Golgi with GDP-mannose, the mannose donor, are essential in *C. albicans*, indicating the overall importance of glycosylation to cellular viability (27-30). However, to date no single glycosylation event in the Golgi has been shown to be essential.

As well as requiring GDP-mannose as the mannose donor Golgi-bound mannosyltransferase enzymes require manganese ions as essential co-factors. In *S. cerevisiae* the P-type ATPase Pmr1p is a Ca\(^{2+}\)/Mn\(^{2+}\) ion pump that supplies the Golgi with these ions (31-33). Homologues of the Pmr1p are classed as secretory pathway Ca\(^{2+}\)-ATPases (SPCA), and are distinct from the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA) and plasma membrane Ca\(^{2+}\)-ATPases (PMCA) (34). The SPCA class of P-type ATPases has been identified in a wide range of fungal and animal cells, including *S. cerevisiae*. Disruption of *PMR1* in *S. cerevisiae* results in a range of phenotypes, including some protein sorting defects, such as the elevated secretion of heterologously expressed proteins (35, 36) and incomplete processing of α-factor (32). The null mutant also has severe glycosylation defects affecting both N- and O-linked mannoproteins (31-33), which are associated with manganese deficiency (33).

To determine the effect of a gross defect in glycosylation on host-fungus interactions and virulence we constructed a null mutant of the *C. albicans* *PMR1* homolog. We reasoned that this mutant would down-regulate glycosylation in general,
allowing the importance of this protein modification to fungal virulence to be assessed. The null mutant had severe defects in both $O$- and $N$-glycosylation and was hypersensitive to cell wall stress. In a murine model of systemic infection the strain was severely attenuated in virulence. These results demonstrate that normal glycosylation is not required for growth in vitro but is essential for virulence of C. albicans.

EXPERIMENTAL PROCEDURES

Strains, Media and Culture Conditions – All strains used and constructed during this work are shown in Table 1. Strains were grown at 30 °C in YEPD (2% (w/v) Mycological Peptone, 1% yeast extract (w/v), 2% glucose (w/v)) or SD (0.67% (w/v) yeast nitrogen base with ammonium sulfate without amino acids, 2% glucose) with uridine (50 µg/ml) as required. Calcium-deficient medium was prepared as SD but with the omission of CaCl$_2$ from yeast nitrogen base. The only source of calcium ions in this medium was from the calcium salt of pantothenoate (present at 0.8 µM). Solid media were prepared with 2% Technical Agar number 3. Hyphal cells were grown in YEPD + 20% newborn calf serum, RPMI-1640 (Invitrogen, Paisley, UK), Lee’s medium pH 6.5 (37) at 37 °C or solid Spider medium (38) at 30 °C. To induce acid phosphatase expression cells were grown in Sabouraud glucose medium depleted for phosphate. Phosphate ions from 1% Neopeptone were allowed to precipitate for 30 min after the addition of 0.01 M MgSO$_4$ and 0.28% NH$_4$OH, followed by filtration and adjustment to pH 5.4 with concentrated HCl before the addition of glucose to a final concentration of 4% (w/v). For virulence testing strains were grown in NGY medium (0.1% Neopeptone, 0.4% glucose and 0.1% yeast extract) at 30 °C.

Construction of Capmr1Δ Null Mutant and Re-integrant Strains – The CaPMRI gene was disrupted by the standard “ura-blaster” protocol (39). To make the disruption cassette the 5’ and 3’ flanking regions of the gene were amplified by PCR (5’ primer pair 5’-GAAGAGCTCACGGCAATGTAAGTAAGTGCG-3’ & 5’-GAAAGATCTGTGATGGCTAATGTGATCG-3’, SacI and BglII restriction sites underlined respectively; 3’ primer pair 5’-GAAGTCGACCAGTATTGCTGATTGAC-3’ & 5’-GAAGCATGCTGCAAGCTATACACCATAAC-3’, SalI and SphI restriction
sites underlined respectively) and cloned into the complementary restriction sites of pMB-7 (39). The disruption cassette was released by digestion with SacI and SphI and contained the URA-blaster cassette flanked by complementary sequences 733 bp upstream and 620 bp downstream of CaPMR1. CaPMR1 was disrupted by sequential rounds of transformation of CAI-4, and the recycling of the URA3 marker by selection on SD plus 5-fluoroorotic acid (5-FOA) (1 mg/ml) and uridine (50 µg/ml). To avoid potential problems associated with the ectopic expression of URA3 (40) the Urα Capmr1Δ null strain was transformed with StuI-digested CIp10 plasmid (41) so that URA3 was expressed at the RPS1 locus (orf19.3002, formally referred to as RPS10). To create a re-integrant strain to act as a control, the CaPMR1 ORF plus 1055 bp of its promoter and 765 bp of terminator were amplified by PCR (Primer pair 5’-CAGACCTAGTCCGACATTGGC-3’ & 5’-ATGAAGCAAGTATCATTGGAGC-3’), and the 3.6 kb product was cloned into pGEM-T Easy (Promega Ltd., Southampton, UK). The insert was released by NotI digestion and subcloned into the NotI site of CIp10. The resulting plasmid was digested with StuI and transformed into the Ura- Capmr1Δ null strain. As a positive control for experiments, CAI-4 was also transformed with StuI-digested CIp10, hence all strains analyzed in this work have URA3 expressed at the RPS1 locus. The two RPS1 alleles in CAI-4 can be distinguished by the presence of a polymorphism resulting in the presence or absence of an XbaI restriction site downstream of RPS1. As an added control, all the strains used were confirmed to have CIp10 inserted into the non-XbaI-containing RPS1 allele.

Sensitivity Testing – To test strains for sensitivity to specific wall-stressing agents strains were initially grown for 24 h in YEPD then washed with water and resuspended at an OD600=1. These cells were then inoculated into YEPD at OD600=0.01 and 95 µl volumes were pipetted into microdilution plate wells. Test agents in 5 µl volumes were added at a range of doubling dilutions. Plates were incubated for 16 h at 30 ºC then absorbance read at 600 nm. All strains were tested in duplicate. The agents tested were; Calcofluor White (100 µg/ml), Congo Red (100 µg/ml), SDS (0.1%), hygromycin B (500 µg/ml), high salt concentration (NaCl, KCl, CaCl2, MnCl2 or MgCl2, all at 1 M), caffeine (50 mM), vanadate (80 mM) and tunicamycin (100 µg/ml). The concentrations listed are the maximum concentration tested for each agent.
Antifungal susceptibility testing was carried out by standard methods (42), except that agents were diluted beyond the stated ranges because derivatives of the *C. albicans* SC5314 genetic background are known to be highly susceptible to antifungal agents.

**Protein Extracts and Western Blotting** – To test for activation of the cell integrity pathway (43) cells were grown in YEPD at 30 ºC and collected in mid-exponential growth. For positive controls the strains were stressed by the addition of 100 µg/ml Calcofluor White 2 h before collection. Cells were washed and resuspended in extraction buffer (100 mM Tris-HCl pH 7.5, 0.01% (w/v) SDS, 1 mM dithiothreitol, 10% (w/v) glycerol and protease inhibitor cocktail (Roche, Lewes, UK)), then disrupted with glass beads in a FastPrep machine (Qbiogene, Cambridge, UK). The lysate was clarified by centrifugation at 21500 x g for 10 min. Protein extracts were quantified by the Coomassie protein assay reagent (Pierce, Cramlington, UK). Prior to western blotting 50 µg of protein was separated on a 4-12% NuPAGE bis-Tris gel (Invitrogen, Paisley, UK) before blotting onto a PVDF membrane. The membrane was blocked in phosphate-buffered saline (PBS) plus 0.1% Tween 20 and 5 mg/ml bovine serum albumin for 2 h at room temperature. Detection was then carried out with the PhosphoPlus p44/42 MAP Kinase antibody kit (New England Biolabs, Hertfordshire, UK) according to manufacturer’s instructions. This antibody cross-reacts with *C. albicans* Mkc1p (Slt2p) in its phosphorylated form.

**Cell Wall Analysis** – Alcian Blue binding assays were carried out as described previously (26). Briefly, approximately 1x10^7 stationary phase cells were washed with water and resuspended in 1 ml of Alcian Blue, (30 µg/ml) in 0.02 M HCl and incubated at room temperature for 10 min. Cells were then spun down and the OD_{620} of the supernatant was measured to determine the level of non-bound Alcian Blue from a calibration curve. From these values the specific amount of Alcian Blue bound to cells (1 U = 1 µg bound/unit OD_{600} of cell suspension) was calculated.

Total cell wall carbohydrate composition was analyzed by hydrolyzing cell walls with sulfuric acid and determining the monosaccharide composition by HPAE-PAD as described previously (44, 45).

**Metabolic Labeling of Glycans and Thin Layer Chromatography (TLC)** – For the analysis of *O*-linked and acid-labile glycans yeast cells were initially labeled with [2-^3^H]-
D-mannose. Cells growing in 2 ml of YP + 0.5% sucrose were incubated with 1.85 MBq of [2-3H]-D-mannose (555 GBq mmol⁻¹; Perkin Elmer, Beaconsfield, UK) at 30 °C for 90 min. Cells were then harvested, resuspended in 100 µl PBS, and disrupted with glass beads as described previously. The cell walls were then collected and washed twice with 1 M NaCl and twice with water. O-linked glycans were released by β-elimination with 100 mM NaOH for 16 h at room temperature. Cell walls were pelleted at 21500 x g for 10 min and the supernatant containing O-linked glycans retained for TLC analysis. The cell walls were washed twice in water then boiled for 1 h in 10 mM HCl to release the acid-labile glycans. Remaining cell walls were then pelleted as previously and the supernatant retained for TLC analysis.

Samples for TLC were spotted onto Silica G-60 TLC plates (Whatman, Brentford, UK) and allowed to dry. Plates were chromatographed by two ascents of the solvent (3:4:2.5:4 ethyl acetate: butan-1-ol: acetic acid: water). For detection, plates were sprayed with En3Hance (Perkin Elmer, Beaconsfield, UK) and visualized by autofluorography (Kodak BioMax XLS).

**Acid Phosphatase Zymogram – In situ** acid phosphatase activity assays were modified from the method of Schweingruber et al (46). Briefly, cells were grown overnight in phosphate-depleted Sabouraud glucose medium to induce acid phosphatase expression. Cells were collected and washed before resuspension in 200 µl lysis buffer (62.5 mM Tris-HCl pH 6.8, 1 mM EDTA, 0.1 mM DTT and protease inhibitor cocktail (Roche, Lewes, UK)). Cells were then disrupted with glass beads as described above. The lysate was clarified by two rounds of centrifugation at 21500 x g for 10 min. For endoglycosidase H (Endo H) treatment the native sample was treated with 25 mU Endo H (Roche, Lewes, UK) in 50 mM sodium acetate pH 5.2 for 16 h at 37 °C. Samples were then mixed with non-denaturing loading dye (62.5 mM Tris-HCl pH 6.8, 0.01% Bromophenol Blue and 15% glycerol) and run on a 6% Tris-glycine PAGE gel (Invitrogen, Paisley, UK) under non-denaturing conditions for 6 h at 125 V. The gel was rinsed in 100 mM sodium acetate pH 5.2 for 10 min at room temperature then incubated with substrate solution (0.05% α-napthyl phosphate in 100 mM sodium acetate pH 5.2) for 30 min at 37 °C. Finally the gel was stained (0.05% α-napthyl phosphate and 0.03%
Fast blue in 100 mM sodium acetate pH5.2) at 60 °C for 10-30 min until the colour developed.

Adhesion to Buccal Epithelial Cells – Adhesion to buccal epithelial cells (BEC) was assessed by the modification of a method previously described for measuring adhesion to vaginal epithelial cells (47). Briefly, BEC were collected from a healthy volunteer, washed twice in physiological saline and resuspended at 5x10^5 BEC/ml. *C. albicans* strains were grown in NGY medium, washed twice in physiological saline and suspended at 5x10^6 cells/ml. The *C. albicans* strain and BEC were then mixed in equal proportions (200 µl), to achieve a 10:1 ratio of yeast cells to BEC, and incubated at 30 °C for 1 h. The cells were fixed with formalin and the BEC scored for the number of yeast cells adhered. At least 150 BEC were assessed for *C. albicans* adherence and the assay was carried out in triplicate.

Virulence Assays – For virulence testing of strains, immunocompetent female BALB/c mice (Harlan Sera-lab, Loughborough, UK) were challenged intravenously. Strains were grown with shaking for 18-24 h in NGY medium at 30 °C. Cells were washed twice in water and resuspended in physiological saline. Groups of 5 or 6 mice were inoculated intravenously with each strain at an inoculum of 2.1x10^4 cfu/g mouse body weight. Mice were monitored over 28 days and animals showing signs of distress or illness were humanely terminated and deaths recorded as occurring the following day. The kidneys and brain were removed post mortem, homogenized in 0.5 ml of water, and *C. albicans* tissue burdens determined by viable counting. All experimentation was carried out under the terms of the UK Home Office licenses for research on animals.

RESULTS

Isolation and analysis of CaPMR1 – We initially identified *CaPMR1* before the completion of the *C. albicans* genome by PCR with primers designed to a unpublished sequence showing homology to *S. cerevisiae* PMR1 (*ScPMR1*) and a degenerate primer to the P-loop found in all P-type ATPases. The regions surrounding *CaPMR1* were then cloned by unidirectional Vectorette PCR (Sigma Genosys, Haverhill, UK) (48). The *CaPMR1* ORF of 2754 bp (EMBL accession AJ277171) is predicted to encode a protein
of 917 amino acids. Subsequently, the \textit{C. albicans} genome sequence has been completed enabling this ORF to be confirmed as the only homolog of \textit{ScPMRI}. As in \textit{S. cerevisiae}, \textit{CaPMRI} is linked to \textit{SUA5}, which is involved in translation initiation. However, the orientation of the two genes is reversed and the conservation of gene order does not extend further in either direction along the chromosome.

The deduced amino acid sequence of \textit{CaPMRI} demonstrated high homology to other secretory pathway P-type ATPases of subfamily IIA (68.7\%, 62.4\%, 62.1\%, 58.3\%, 57.6\%, 51.8\% and 51.5\% identity to Pmr1p of \textit{Pichia angusta}, \textit{Kluyveromyces lactis}, \textit{S. cerevisiae}, \textit{Yarrowia lipolytica}, and \textit{Schizosaccharomyces pombe} PGAK2p, human ATP2C1p and rat ATP2C1p respectively). Nine putative transmembrane regions are predicted by hydrophobicity profile analysis (http://www.cbs.dtu.dk/services/TMHMM/) and by the similarity of this profile to other Ca\textsuperscript{2+} P-type ATPases. As with other Ca\textsuperscript{2+} P-type ATPases the transmembrane domains are clustered into two groups separated by a central hydrophilic loop. All 10 conserved regions (a-j) that characterize the P-type ATPase family (49) are maintained in \textit{CaPMRI} (Fig. 1). In particular, region f contains the aspartic acid residue in the D\textsubscript{349}KTGTLT motif that is the phosphorylation site in P-type ATPases (34). Region c contains the T\textsubscript{189}GE motif believed to be important in removing the phosphate group from the enzyme intermediate. Regions g and i, responsible for ATP binding, are also well conserved.

Deletion of \textit{CaPMRI} – \textit{CaPMRI} was disrupted in strain CAI-4 by the standard “ura-blaster” method (39). This involved the deletion of the central 1803 bp region containing all 10 regions characteristic of P-type ATPases. To avoid potential problems with ectopic expression of \textit{URA3} the Ura\textsuperscript{-} derivatives were transformed with plasmid C\textsuperscript{10}, so that \textit{URA3} is expressed from the neutral \textit{RPS1} locus (40, 41). A re-integrant strain was also constructed where \textit{CaPMRI} was introduced, under the control of its own promoter, into the \textit{Capmr1\Delta} null mutant at the \textit{RPS1} locus. Strain CAI-4, transformed with C\textsuperscript{10}, was used as a control in all experiments.

Deletion of \textit{CaPMRI} had no direct effect on growth rate in either YEPD or minimal medium (SD). However the yeast cells in liquid medium tended to form small aggregates (Fig. 2A). There was also no obvious defect in hypha formation in response to serum, RPMI-1640 or Lee’s medium at pH 6.5. However, there was a delay in filament
formation on solid Spider medium (Fig. 2B.). Both the small cellular aggregates and the delay in filament formation on Spider medium were restored to wild type in the re-integrant strain. Hence growth in vitro was not markedly affected in the Capmr1Δ null mutant.

*Calcium-related growth defects in Capmr1Δ* - As mentioned above, deletion of CaPMR1 had no effect on the growth rate of the strain in laboratory media. Manganese ions are normally present only at trace concentrations below 10 µM in laboratory media and cannot be depleted further without chelation. Therefore growth parameters were initially assessed under Ca2+-depleted conditions alone. Even when the Capmr1Δ mutant was grown in Ca2+-depleted medium there was no obvious effect on growth rate (Fig. 3A). The only source of Ca2+ in this medium was from the calcium salt of pantothenoate (at 0.8 µM) and contaminants. However, when viable cell counts were measured it was clear that the null mutant started to lose viability as the cells entered stationary phase. After 72 h of growth in Ca2+-depleted medium the null mutant’s viability dropped over 1000-fold (Fig. 3B). When the null mutant was grown in standard SD medium (Ca2+ ~ 1 mM) there was also a slight decrease in viability at 72 h. Loss of viability was not detected when the strain was grown in SD medium supplemented with 10 mM CaCl2. The wild type and re-integrant strains displayed no loss of viability when grown on SD medium at any of the Ca2+ levels tested.

The Capmr1Δ null mutant was also hypersensitive to EGTA. Growth of the null mutant was completely blocked by addition of 15 mM EGTA, whereas the wild type and re-integrant strains were unaffected (Fig. 4). This hypersensitivity could be reversed by the addition of either 10 mM Ca2+ or Mn2+, but not by equivalent concentrations of Mg2+, Na+ or K+. Unlike in S. cerevisiae, deletion of CaPMR1 did not alter sensitivity to the calmodulin inhibitor trifluoperazine, nor did it affect the level of manganese toxicity (not shown).

*Glycosylation Defects in the Capmr1Δ Mutant –* CaPMR1 provides the Golgi with manganese ions that are required as a co-factor for the Golgi resident mannosyltransferases. We therefore analyzed the extent of N- and O-linked glycosylation in the Capmr1Δ null mutant. We assessed the electrophoretic mobility of secreted acid phosphatase, which is known to be heavily N-glycosylated, by activity
staining (50). The mobility of acid phosphatase present in protein extracts from the 
Capmr1Δ null mutant was increased in the native gel (Fig. 5A). Hence the null mutant had an N-glycosylation defect. After treatment with Endo H to remove the N-linked side chains the acid phosphatase migrated faster through the native gel; however, it still appeared as a diffuse band, presumably due to variations in O-linked glycosylation. In this case there was no difference between the null mutant, wild type and re-integrant controls. However, because O-linked side chains are short, this method cannot be used to assess O-glycosylation defects.

N-linked glycosylated side chains contain the acid-labile fraction phosphomannan, which is incorporated as the protein passes through the Golgi. This fraction provides the cell wall with its negative charge and can be easily detected with the cationic dye Alcian Blue. The Capmr1Δ null mutant demonstrated a severe reduction in specific Alcian Blue binding from 143 (±6.7) U in the wild type strain to 6.7 (±3.8) U in the null mutant (5% of wild type). The re-integrant strain had an intermediate Alcian Blue binding phenotype of 102 (±7.5) U (74% of wild type). We also analyzed the acid-labile phosphomannan fraction directly by TLC. Cells were labeled with [2-3H]-D-mannose and phosphomannan was isolated by HCl treatment of cell walls. The TLC autofluorogram clearly demonstrated that the Capmr1Δ null mutant was almost completely devoid of phosphomannan (Fig. 5B.). The wild type and re-integrant strains displayed normal phosphomannan of 1 to 8 β1,2-linked mannose residues (26, 51).

The consequence of loss of CaPMR1 on the O-mannan structure was also assessed by TLC analysis. O-mannan of C. albicans typically comprises 1 to 5 α1,2-linked mannose residues, the first of which is added in the ER and subsequent residues are added in the Golgi. The TLC analysis clearly demonstrated that O-mannan was truncated in the Capmr1Δ null mutant (Fig. 5C), with almost no detectable Man3 to Man5. The fact that Man2 was present on the O-mannan isolated implies that some Golgi-based mannosyltransferase activity is retained in the Capmr1Δ null mutant. Re-integration of CaPMR1 to the null mutant restored the normal O-mannan structure.

To confirm the gross glycosylation defect in the Capmr1Δ null mutant and to determine the effect on the cell wall we analyzed wall composition by HPAE-PAD. This method determines the carbohydrate composition of the wall by analyzing its
monosaccharide composition where glucose, mannose and glucosamine are detected. These levels related to the presence of glucan, mannan and chitin in the cell wall respectively. This demonstrated a clear defect in mannan levels, which dropped from 39% of the cell wall in the wild type strain to only 8% in the \textit{Capmr1}Δ null mutant (80% reduction). This decrease was reciprocated with an increase in glucan levels from 60% in the wild type to 90% in the null mutant. The level of chitin in the strains was unchanged.

\textit{Cell wall Sensitivity and Cell Integrity Pathway Activation} – To test the effect of deleting \textit{CaPMRI} on the integrity of the cell wall we tested the null mutant for sensitivity to a range of cell wall perturbing agents and other agents whose effects have been associated with altered cell wall and glycosylation. The \textit{Capmr1}Δ null mutant was clearly hypersensitive to the cell wall perturbing agents Calcofluor White and Congo Red and displayed increased sensitivity to hygromycin B (Fig. 6), a phenotype commonly seen in glycosylation mutants (52). However, there was no change in the level of sensitivity towards other agents such as SDS, caffeine, vanadate, tunicamycin, NaCl and KCl. Antifungal susceptibility testing demonstrated that the null mutant showed no change in sensitivity towards antifungal agents in clinical use, such as the azoles (fluconazole,itraconazole and ketoconazole), flucytosine, amphotericin B, terbinafine, or the β1,3-glucan synthase inhibitor caspofungin (data not shown).

The hypersensitivity of the \textit{Capmr1}Δ null mutant to cell wall-perturbing agents shows that the wall is altered and is more sensitive to stress. Therefore, we determined whether the cell integrity pathway was induced in the mutant. The cell integrity pathway signals through Pkc1p and results in the phosphorylation of the MAP kinase Mkc1p (43). We tested the activation of Mkc1p in our strains by western blotting with a commercially available antibody that binds to phosphorylated-Mkc1p. The \textit{Capmr1}Δ null mutant had an activated Mkc1p during exponential growth, whereas Mkc1p was not activated in the wild type or re-integrant controls (Fig. 7). As a control the strains were also stressed with 100 µg/ml Calcofluor White for 2 h. This again demonstrated activation of the cell integrity pathway. The results of sensitivity testing and the constitutive activation of the cell wall integrity pathway show that the \textit{Capmr1}Δ null mutant has a defective cell wall.

\textit{Adhesion of the Capmr1Δ null mutant} – Mannoproteins have been implicated in \textit{C. albicans} adhesion hence we tested the ability of the \textit{Capmr1}Δ mutant to adhere to
buccal epithelial cells (BEC). Initially it appeared that there was an increase in adherence from 0.38 ± 0.03 adhered cells/BEC in the wild type control compared to 1.19 ± 0.22 adhered cells/BEC in the null mutant. However, the null mutant grows as small clumps of cells (Fig. 2A.) and this increase in adhesion may reflect the null mutant clumping phenotype. Indeed, the number of BEC with one or more adherent yeast cells did not increase (76 ± 1.4% negative for wild type, 74 ± 4.5% negative for null mutant), suggesting that adhesion was not significantly altered.

*Capmr1Δ* Null Mutant is Attenuated in Virulence – The virulence of the *Capmr1Δ* null mutant and relevant control strains was tested in a mouse model of systemic infection. The *Capmr1Δ* null mutant was highly attenuated in virulence with all mice surviving until the end of the experiment, whereas challenge with the wild type control demonstrated a mean survival time of 7 days (log rank test; P<0.05) (Fig.8, Table 2). The re-integrant strain demonstrated an intermediate phenotype, with a mean survival time of 13.8 days that was significantly different from the null mutant (log rank test; P<0.05). The intermediate phenotype is suggestive of a gene dosage effect on virulence. Such effects are typical of other heterozygous and re-integrant strains containing a single wild type copy of the target gene (53-55). In terms of tissue burdens determined for kidneys and brains of infected mice, the wild type and re-integrant strain were not significantly different. The *Capmr1Δ* null mutant demonstrated a clear reduction in tissue burdens, with a greater than 2-log reduction in kidney cfu/g and 1-log reduction in brain cfu/g (Table 2). There were also a significant number of *Candida*-negative organs: 33% of kidneys and 50% of brains with the *Capmr1Δ* mutant. Hence, although the *Capmr1Δ* mutant was not affected in growth *in vitro* this null mutant was extremely attenuated in virulence in a mouse model.

**DISCUSSION**

In this study we have analyzed the effect of a gross defect in glycosylation in *C. albicans* by creating a null mutant in *PMR1*, which is required for glycosylation in the Golgi. Pmr1p is a P-type ATPase and supplies the Golgi body with calcium and manganese ions. In the Golgi, manganese ions are required as an essential co-factor for
mannosyltransferases. Hence by disrupting \textit{PMR1} we could determine the effect of a general defect in glycosylation both at the cellular level and in pathogenesis. The \textit{Capmr1}Δ null mutant clearly demonstrated a general glycosylation defect, associated cell wall changes, and was severely attenuated in virulence in a murine model of systemic infection.

Previous studies have also examined gross glycosylation defects by studying the synthesis (29) and transport (27) of GDP-mannose into the Golgi, where it acts as the mannose donor for mannosyltransferases. Total lack of GDP-mannose would not only block glycosylation in the Golgi but also the essential processes of \(O\)-glycosylation and biosynthesis of the GPI-anchor in the ER, as these steps require dolichyl-phosphomannose that is synthesized from GDP-mannose. Hence, \textit{CaSRB1}, which synthesizes GDP-mannose, was found to be essential and its depletion to have pleiotropic effects (29). The Golgi GDP-mannose transporter, \textit{CaVRG4}, is essential in \textit{C. albicans} (27) and \textit{S. cerevisiae} (56, 57). This suggests that a complete lack of glycosylation in the Golgi results in non-viability. The essential nature of both \textit{CaSRB1} and \textit{CaVRG4}, whilst emphasizing the importance of glycosylation, means that the role of the glycans in host-fungal interactions and virulence cannot be assessed. The Golgi GDPase \textit{CaGDA1} has also been studied (30); this enzyme converts GDP to GMP which is then exported from the Golgi by Vrg4p in an antiport process with GDP-mannose. However, \textit{CaGDA1} is non–essential, presumably due to functional redundancy, and does not exhibit a gross glycosylation defect. It does demonstrate a partial defect in \(O\)-glycosylation and, as visualized by Alcian Blue binding, has a slight reduction in phosphomannan; however, it displays no defect in the acid-stable \(N\)-glycans.

In \textit{S. cerevisiae}, loss of \textit{PMR1} resulted in a strain displaying a slow-growth phenotype that could be alleviated by adding exogenous calcium ions to the medium. However, higher \(Ca^{2+}\) concentrations in the medium resulted in the strain losing viability more quickly upon entering stationary phase (31). In our studies, deletion of \textit{CaPMR1} had no effect on the growth rate of the strain, even when grown on calcium-depleted medium. Additionally, the viability phenotype was the opposite to that seen in \textit{S. cerevisiae}, in that the \textit{Capmr1}Δ null mutant lost viability upon entering stationary phase after growth on \(Ca^{2+}\)-depleted medium. Therefore, there must be subtle differences
in the homeostasis of calcium ions in *S. cerevisiae* and *C. albicans*. Unlike in *S. cerevisiae*, we also saw no change in the level of manganese toxicity towards the *Capmr1Δ* null mutant. Therefore, there are also differences in the tolerance of *S. cerevisiae* and *C. albicans* to manganese ions.

The observed calcium-dependent drop in viability of the null mutant after entering stationary phase may affect pathogenicity. Calcium ions are present in serum at 95 µg/ml of which 48 µg/ml (~1.2 mM) is available in an accessible ionized form (58). This concentration approximates that present in standard SD medium, where only a slight decrease in long-term viability was seen (~10% drop at 72 h). Therefore, although we cannot rule out that this may have influenced the virulence study it is likely to only be a minor effect at physiological calcium and manganese ion levels, and as such could not account for the almost complete loss of virulence in the *Capmr1Δ* mutant.

Work in *Y. lipolytica* has shown that loss of Pmr1p can affect secretion. Depending on the marker protein analyzed, secretion was either unchanged, enhanced or decreased (59). Loss of Pmr1p can also affect the secretion of proteins expressed heterologously in *S. cerevisiae* and *K. lactis* (35, 36, 60). We tested the level of secretion in the *Capmr1Δ* null mutant by analyzing the activity of cell-associated acid phosphatase and the secreted aspartyl proteinase (SAP) (data not shown). We detected a slight decrease in the level of cell-associated acid phosphatase activity, 70% (±2.4) of the wild type, and a slight increase in the level of SAP activity, 163% (±3.7) of wild type. Therefore, the changes in secretion detected in the *Capmr1Δ* null mutant were not as high as those reported for other organisms. However, it was clear from these published reports that the levels of change detected are dependant on the protein that is secreted. Therefore we cannot rule out that proteins other than cell-associated acid phosphatase and the secreted aspartyl proteinase may be more severely affected in the *Capmr1Δ* null mutant.

The *Capmr1Δ* null mutant had a clumpy phenotype, with most cells present in small aggregates. This may be due to changes in the cell wall composition resulting in flocculation. However, examination of the clumps suggests that they may be the result of a cell separation defect. This would suggest that daughter cells are delayed in cell wall separation, perhaps due to a decrease in the activity of cell wall-hydrolyzing enzymes. A similar clumping phenotype has been reported for other *C. albicans* glycosylation
mutants, such as in the Camnt1Δ/Camnt2Δ double mutant (19). A more severe clumping phenotype was apparent in the Camnnn9Δ and Cavrg4Δ mutants (25, 61). A severe cytokinesis defect in a *S. pombe* pmr1Δ mutant has also been reported recently (62). The clumping phenotype posed technical difficulties with *in vitro* assays of adhesion. However, it was clear that the overall number of epithelial cells adhered to was unaffected with the mutant, suggesting that loss of Pmr1p did not have a marked effect on adhesion.

Evidence was found for a gross defect in glycosylation in the Capmr1Δ null mutant, with *N*-glycosylation severely affected as demonstrated by the under-glycosylation of acid phosphatase and the almost complete absence of phosphomannan (Fig. 5). *O*-glycosylation was also affected such that *O*-mannan was truncated with a marked reduction in Man$_3$ to Man$_5$ (Fig. 5). The observation that Man$_2$ was still present in the *O*-mannan isolated from the Capmr1Δ null mutant implies that some mannosyltransferases are still active in the Golgi. This may reflect the localization of specific mannosyltransferases within the Golgi, where enzymes of the *cis*-Golgi may receive manganese from vesicles derived from the ER. Therefore, in the Capmr1Δ null mutant Man$_2$ may have been present because it was added in an early Golgi compartment that received manganese ions from ER-derived vesicles. This would also suggest that phosphomannan, which is almost completely absent in the Capmr1Δ null mutant, is a component added later in the Golgi network, and as such is more sensitive to the loss of Pmr1p. Alternatively the presence of Man$_2$ in the *O*-mannan of the Capmr1Δ null mutant may reflect the affinity of specific mannosyltransferases for manganese ions. Those that have the highest cation affinity may remain most active in a manganese-limited compartment.

The cell wall is significantly altered in the Capmr1Δ null mutant, exemplified by the 80% drop in mannose content that correlates with the gross glycosylation defect. Consequences of the loss of Pmr1p were significant enough to result in the constitutive activation of the cell integrity pathway acting to compensate for the loss of mannan. The null mutant was hypersensitive to the cell wall perturbing agents Calcofluor White and Congo Red, which bind chitin and glucan, and interfere with their synthesis and cross-linking. Increased sensitivity was also seen to hygromycin B, a phenotype characteristic
of N-glycosylation mutants in *S. cerevisiae* (52). However, no change in sensitivity was detected for vanadate where resistance is linked with N-glycosylation mutants in *S. cerevisiae* (57, 63). There was also no change in sensitivity to SDS that would target both cell wall proteins and the plasma membrane, or to high salt conditions, indicating that the mutant was not osmotically fragile.

The *Capmr1Δ* null mutant displayed no significant growth defect *in vitro*, yet had an altered cell wall due to the gross glycosylation defect. This allowed us to assess the importance to virulence of glycosylation events that occur in the Golgi. The null mutant was highly attenuated in virulence with all mice surviving the course of the experiment and displaying a significant drop in organ burdens. The low organ burdens and the number of *Candida*-negative organs suggest that either the host was able effectively to prevent colonization or that the mutant was unable to reach deep-seated infection sites. These results underline the importance of glycosylation, both to the cell wall architecture and pathogenesis of *C. albicans* disease. Questions still remain on the identification of specific epitopes involved in host-pathogen interactions, although in terms of carbohydrate epitopes recent work has confirmed the importance of *O*-glycans (19) and down-played the importance of phosphomannan (26). Continued research into the construction, modification and presentation of specific mannan epitopes should ultimately provide answers to these questions.

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   416, 203-206
   Microbiol. 47, 1029-1043


FIGURE LEGENDS

FIG. 1. Amino acid conservation in the 10 regions (a-j) present in all P-type ATPases. Numbers indicate the amino acid sequence position. Sequences present are; ScPMR1, S. cerevisiae; YlPMR1, Yarrowia lipolytica; KlPMR1, Kluyveromyces lactis; PaPMR1, Pichia angusta; SpPGAK2, Schizosaccharomyces pombe; and HsATP2C1, Homo sapiens.

FIG. 2. Cell and colony morphology of the Capmr1Δ null mutant. A, Cell morphology after 16 h growth in YEPD medium at 30 ºC demonstrating clumping of cells. Scale bar, 10 µm. B, Colony morphology after 5 days growth at 30 ºC on Solid Spider Medium displaying a delay of filament formation in the null mutant. Scale bar, 1 mm.

FIG. 3. Growth and viability affects in Ca²⁺-defined medium. Wild type (closed squares), Capmr1Δ null mutant (open squares) and the re-integrant strain (open triangles) were grown in minimal medium (MM, 1 mM Ca²⁺), calcium depleted MM (MM-Ca²⁺, ~1 µM Ca²⁺) or calcium enriched MM (MM+Ca²⁺, 10 mM Ca²⁺). Samples were taken at set time points and growth and viability assessed by optical density (OD₆₀₀, A) and viable cell counts by plating onto YEPD (B).

FIG. 4. EGTA hypersensitivity of the Capmr1Δ null mutant. A, Growth of the wild type (PMR1), null mutant (pmr1Δ) and re-integrant (pmr1Δ+PMR1) was scored after 2 days growth on minimal medium (MM) or MM + 15 mM EGTA. Hypersensitivity to EGTA could be reversed by the addition of 10 mM CaCl₂ or MnCl₂ but not MgCl₂. B, EGTA sensitivity was quantified by optical density (OD₆₀₀) after 16 h growth in YEPD with varying EGTA concentrations; wild type (closed squares), null mutant (open squares) and the re-integrant (open triangles). Errors are S.D.

FIG. 5. Glycosylation defects in the Capmr1Δ null mutant. Glycosylation status was assessed in 1. wild type, 2. Capmr1Δ null mutant and 3. re-integrant strains. A, The level of N-glycosylation was determined by activity staining for acid phosphatase. Protein samples from the strains were separated on non-denaturing PAGE gels for 6 h and
colorimetrically stained for acid phosphatase activity. Samples were also treated with endoglycosidase H (EndoH) to remove N-glycans. B, TLC analysis of acid-labile phosphomannan glycans. Acid hydrolysis products from cell wall of [2-^3^H] mannose labeled cells were separated on a Silica G-60 TLC plate and exposed to film. C, TLC analysis of β-eliminated O-glycans. [2-^3^H] mannose labeled cell walls were β-eliminated by incubating in 100 mM NaOH for 16 h, and glycan samples separated by TLC analysis. Each lane was loaded with glycans extracted from approximately the same number of cells. The position of mannose oligomers are shown M1-M5 (mannose to manno-pentose), the position of the origin for TLC analysis is indicated (Or).

**Fig. 6.** *Capmr1Δ* null mutant displays sensitivity to cell wall perturbing agents. Sensitivity of the wild type (closed squares), null mutant (open squares) and re-integrant (open triangles) strains to cell wall perturbing agents was determined quantitatively by a broth dilution method (see experimental procedures). The agents where the *Capmr1Δ* null mutant demonstrated hypersensitivity are shown (Calcofluor White, Congo Red and Hygromycin B). No change in sensitivity was seen for SDS, caffeine, vanadate, tunicamycin, NaCl or KCl (not shown). Errors are S.D.

**Fig. 7.** Constitutive activation of the cell wall integrity pathway in the *Capmr1Δ* null mutant. Phosphorylation status of CaMkc1p was determined by western blotting using the PhosphoPlus p44/42 MAP kinase antibody against protein extracts prepared from strains growing in mid-exponential phase in the presence or absence of 100 µg/ml Calcofluor White. Strains tested were 1. Wild type, 2. *Capmr1Δ* null mutant and 3. re-integrant. Equal loading was verified by Ponceau S staining and the intensity of non-specific bands.

**Fig. 8.** Attenuation of virulence in the *Capmr1Δ* null mutant. The wild type (closed squares), null mutant (open squares) and re-integrant (open triangles) strains were tested for virulence in a mouse model of systemic infection. Mice (n=6 for wild type and null mutant, n=5 for re-integrant) were challenged with the strains at an inoculum of 2.1x10^4 cfu/g body weight.
Table 1.
Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAI-4</td>
<td>-</td>
<td>\textit{ura3}\Delta::imm434/\textit{ura3}\Delta::imm434</td>
<td>(39)</td>
</tr>
<tr>
<td>NGY152</td>
<td>CAI-4</td>
<td>As CAI-4 but \textit{RPS1/rps1}\Delta::Clp10</td>
<td>(40)</td>
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<tr>
<td>NGY95</td>
<td>CAI-4</td>
<td>As CAI-4 but \textit{PMR1/pmr1}\Delta::\textit{hisG-URA3-hisG}</td>
<td>This study</td>
</tr>
<tr>
<td>NGY96</td>
<td>NGY95</td>
<td>As CAI-4 but \textit{PMR1/pmr1}\Delta::\textit{hisG}</td>
<td>This study</td>
</tr>
<tr>
<td>NGY97</td>
<td>NGY96</td>
<td>As CAI-4 but \textit{pmr1}\Delta::\textit{hisG/pmr1}\Delta::\textit{hisG-URA3-hisG}</td>
<td>This study</td>
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<tr>
<td>NGY98</td>
<td>NGY97</td>
<td>As CAI-4 but \textit{pmr1}\Delta::\textit{hisG/pmr1}\Delta::\textit{hisG}</td>
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</tr>
<tr>
<td>NGY355</td>
<td>NGY98</td>
<td>As CAI-4 but \textit{pmr1}\Delta::\textit{hisG/pmr1}\Delta::\textit{hisG}, \textit{RPS1/rps1}\Delta::Clp10</td>
<td>This study</td>
</tr>
<tr>
<td>NGY356</td>
<td>NGY98</td>
<td>As CAI-4 but \textit{pmr1}\Delta::\textit{hisG/pmr1}\Delta::\textit{hisG}, \textit{RPS1/rps1}\Delta::Clp10-\textit{PMR1}</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2.

Mean survival times and organ burden for BALB/c mice infected with Capmr1Δ null mutant and control strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean survival (days ± SD)</th>
<th>Kidney burden (log_{10}cfu/g ± SD)</th>
<th>Negative kidneys</th>
<th>Brain burden (log_{10}cfu/g ± SD)</th>
<th>Negative brains</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaPMR1</td>
<td>7 ± 0</td>
<td>6.2 ± 0.5</td>
<td>0%</td>
<td>3.2 ± 1.0</td>
<td>0%</td>
</tr>
<tr>
<td>Capmr1Δ</td>
<td>28 ± 0</td>
<td>3.6 ± 2.2</td>
<td>33%</td>
<td>1.9 ± 0.6</td>
<td>50%</td>
</tr>
<tr>
<td>Capmr1Δ+PMR1</td>
<td>13.8 ± 4.3</td>
<td>6.0 ± 0.6</td>
<td>0%</td>
<td>2.7 ± 0.5</td>
<td>0%</td>
</tr>
</tbody>
</table>
Fig. 2. Bates et al.
Fig. 3. Bates et al.
Fig. 4. Bates et al.
Fig. 5. Bates et. al.
Fig. 6. Bates et al.
Fig. 7. Bates et. al.
Fig. 8. Bates et. al.
Candida albicans Pmr1p, a secretory pathway Ca\textsuperscript{2+}/Mn\textsuperscript{2+} P-type ATPase, is required for glycosylation and virulence

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